Molecular Biology of the Cell
Vol. 8, 719–728, April 1997

Cytoplasmic Tails of β_1, β_2, and β_7 Integrins Differentially Regulate LFA-1 Function in K562 Cells

Marijke Lub,* Sandra J. van Vliet,* Sigrid P.M.A. Oomen,* Reagan A. Pieters,* Martyn Robinson,† Carl G. Figdor,* and Yvette van Kooyk*‡

*Department of Tumor Immunology, University Hospital Nijmegen St. Radboud, Nijmegen, the Netherlands; and †Department of Exploratory Research, Celltech, United Kingdom

Submitted September 29, 1996; Accepted December 18, 1996
Monitoring Editor: Richard Hynes

The β_2 integrin lymphocyte function-associated antigen 1 (LFA-1) mediates activation-dependent adhesion of lymphocytes. To investigate whether lymphocyte-specific elements are essential for LFA-1 function, we expressed LFA-1 in the erythroleukemic cell line K562, which expresses only the integrin very late antigen 5. We observed that LFA-1-expressing K562 cannot bind to intercellular adhesion molecule 1-coated surfaces when stimulated by phorbol 12-myristate 13-acetate (PMA), whereas the LFA-1-activating antibody KIM185 markedly enhanced adhesion. Because the endogenously expressed β_1 integrin very late antigen 5 is readily activated by PMA, we investigated the role of the cytoplasmic domain of distinct β subunits in regulating LFA-1 function. Transfection of chimeric LFA-1 receptors in K562 cells reveals that replacement of the β_2 cytoplasmic tail with the β_1 but not the β_7 cytoplasmic tail completely restores PMA responsiveness of LFA-1, whereas a β_2 cytoplasmic deletion mutant of LFA-1 is constitutively active. Both deletion of the β_2 cytoplasmic tail or replacement by the β_1 cytoplasmic tail alters the localization of LFA-1 into clusters, thereby regulating LFA-1 activation and LFA-1-mediated adhesion to intercellular adhesion molecule 1. These data demonstrate that distinct signaling routes activate β_1 and β_2 integrins through the β-chain and hint at the involvement of lymphocyte-specific signal transduction elements in β_2 and β_7 integrin activation that are absent in the nonlymphocytic cell line K562.

INTRODUCTION

The β_2 integrin lymphocyte function-associated antigen 1 (LFA-1;[1] CD11a/CD18 or $\alpha_L\beta_2$) is a lymphocyte-specific adhesion receptor that coordinates different adhesive and signaling interactions within the immune system (Kurzinger *et al.*, 1981; Martz, 1987; Arnaout, 1990; Springer, 1990). LFA-1 mediates cell-cell adhesion upon binding to any one of its ligands, which are intercellular adhesion molecule (ICAM) 1 (Marlin and Springer, 1987), ICAM-2 (Staunton *et al.*, 1989), and ICAM-3 (de Fougerolles and Springer, 1992; Fawcett *et al.*, 1992; Vazeux *et al.*, 1992; de Fougerolles *et al.*, 1993). Integrins are heterodimeric transmembrane molecules composed of an α-chain that is noncovalently linked to a β-chain. Various integrin subfamilies can be distinguished by differences in their β-chains (Hynes, 1987).

Besides β_2 integrins, lymphocytes also express β_1 and β_7 integrins. Similar to the β_2 integrins, $\alpha_4\beta_7$ is only expressed on lymphocytes and mediates adhesion to vascular cell adhesion molecule 1, muscosal addressing cell adhesion molecule, α_4, or the extracellular matrix component fibronectin (Ruegg *et al.*, 1992; Altevogt *et al.*, 1995; Berlin *et al.*, 1995). In contrast, the β_1 integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$ are not lymphocyte-specific adhesion receptors, since they are found on a variety of other cell types (Hemler, 1988, 1990; Hemler *et al.*,

‡ Corresponding author: Department of Tumor Immunology, University Hospital Nijmegen St Radboud, Philips van Leydenlaan 25, 6525 EX Nijmegen, the Netherlands.

[1] Abbreviations used: ICAM, intercellular adhesion molecule; LFA-1, lymphocyte function-associated antigen 1; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate.

1990). $\alpha_4\beta_1$ mediates adhesion to VCAM-1, α_4, and the extracellular matrix component fibronectin, whereas $\alpha_5\beta_1$, predominantly binds to fibronectin (Hemler *et al.*, 1987; Takada *et al.*, 1987; Hemler *et al.*, 1988; Altevogt *et al.*, 1995).

LFA-1/ICAM-1 adhesion requires activation of LFA-1 through intracellular signals (Martz, 1987; Dustin and Springer, 1989; van Kooyk *et al.*, 1989; Hynes, 1992). This activation process, termed "inside-out" signaling, is common to all integrins including the β_1 and β_7 integrins (Keizer *et al.*, 1988; Dustin and Springer, 1989; van Kooyk *et al.*, 1989; Altieri, 1991; Dransfield *et al.*, 1992b; Andrew *et al.*, 1993; Landis *et al.*, 1993). Activation of LFA-1 is thought to result in a conformational change in the α/β heterodimer, leading to an enhanced binding affinity LFA-1 for its ligand ICAM-1 (Lollo *et al.*, 1993). Both affinity (active conformation) and avidity (clustering) changes are required to obtain strong binding of LFA-1 to ICAM-1 (Dustin, 1990). Affinity/avidity changes in LFA-1 depend on an intact cytoskeleton, physiological temperature, and binding of divalent cations, in particular Mg^{2+} (Rothlein and Springer, 1986; Dransfield and Hogg, 1989; Figdor *et al.*, 1990; Dransfield *et al.*, 1992a). Binding of Ca^{2+} to LFA-1 supports clustering (high-avidity state) of LFA-1 on the cell surface, resulting in enhanced LFA-1-mediated adhesion (Figdor *et al.*, 1990; van Kooyk *et al.*, 1994).

Alternatively, binding of certain activating anti-integrin antibodies, or the divalent cation Mn^{2+}, to the extracellular part of the integrin, can also induce an active conformation (high-affinity state) of the (β_1, β_2, β_7) integrin, resulting in increased adhesion to the ligand (Keizer *et al.*, 1988; Robinson *et al.*, 1992; Andrew *et al.*, 1993; Landis *et al.*, 1993). It is thought that these activating anti-integrin monoclonal antibodies (mAbs) mimic ligand binding and stimulate postligand binding signaling ("outside-in" signaling). Outside-in signaling generates different intracellular signals, including phosphorylation of distinct tyrosine kinases and other proteins (Hynes, 1992; Kanner *et al.*, 1993; Arroyo *et al.*, 1994).

Although the cytoplasmic tail of the α- and β-chains of β_1, β_2, and β_7 integrins are relatively short (46, 45, and 51 amino acids for the β_1, β_2, and β_7 cytoplasmic tail, respectively) and do not contain any intrinsic kinase activity, the cytoplasmic tails seem to be involved in transmitting inside-out signals as well as outside-in signals to and from the integrin molecule. It has been demonstrated that the adhesiveness of LFA-1 is controlled by the cytoplasmic domain of the β_2 subunit, because truncation of the cytoplasmic β_2 tail, but not the α_L tail, eliminates LFA-1 binding to ICAM-1 (Hibbs *et al.*, 1991b). In particular, mutations of a triplet of threonines (positions 758–760) and the phenylalanine residue at position 766, in the β_2 cytoplasmic tail profoundly reduced the adhesiveness of LFA-1 (Hibbs *et al.*, 1991a; Peter and O'Toole, 1995). It has been suggested that the altered adhesiveness due to mutation of the threonine triplet is caused by an altered cytoskeletal association/organization and not to an affinity change in LFA-1 (Peter and O'Toole, 1995). Because deletion of the cytoplasmic domain of the α_L subunit does not affect binding to ICAM-1, it is hypothesized that the cytoplasmic tail of α_L is predominantly involved in "postligand binding" events of this integrin (Hibbs *et al.*, 1991b).

Similarly, truncation of the cytoplasmic domain of the β_1 integrin subunit impairs adhesion to both fibronectin and laminin and has been shown to be important for cell spreading and localization to focal contacts (Solowska *et al.*, 1989; Hayashi *et al.*, 1990; Marcantonio *et al.*, 1990; Reszka *et al.*, 1992). Moreover, also partial removal of the β_7 cytoplasmic domain displayed no ligand binding activity of $\alpha_4\beta_7$ (Crowe *et al.*, 1994).

In this study, we investigated whether a lymphocytic environment is required to mediate adhesion through the β_2 integrin LFA-1 and whether this depends on the cytoplasmic tail of the β-chain. Therefore, we used the erythroleukemic K562 cells to express either wild-type LFA-1, LFA-1 in which the entire cytoplasmic tail of the β_2 was deleted (Δ724), or LFA-1 in which the cytoplasmic domain of the β_2 was exchanged for the cytoplasmic domains of β_1 or β_7. We demonstrate that the cytoplasmic tail of the β-chain of integrins plays a pivotal role in regulating ligand binding affinity (active conformation) and avidity (clustering) and suggest that K562 and lymphocytes use different signaling elements to activate integrins.

MATERIALS AND METHODS

mAbs

mAbs SPV-L7 (IgG1), NKI-L15 (IgG2a), and NKI-L16 (IgG2a) reactive with the α-chain of LFA-1 were raised as described previously (Keizer *et al.*, 1985, 1988). The nonblocking mAb TS2/4 (IgG1) reactive with α_L was provided by Dr. E Martz (Sanchez Madrid *et al.*, 1982). mAb 60.3 (IgG1), directed against β_2 was obtained from Dr. J.M. Harlan (Beatty *et al.*, 1983). The anti-β_2 mAb KIM185 (IgG1) was used to activate β_2 integrins (Andrew *et al.*, 1993) and the anti-β_1 mAb TS2/16 to activate β_1 integrins (Hemler *et al.*, 1984; van de Wiel-van Kemenade *et al.*, 1992). The anti-α_5 mAb SAM-1 (IgG1) was used to block very late antigen 5-dependent adhesion (Keizer *et al.*, 1987).

DNA Constructs

The 4.2-kb α-chain of LFA-1 was cloned in the *Xba*I site of the pCDM8 vector that directs expression of α_L from the cytomegalovirus (CMV) AD169 immediate early promotor (pCDL1). The 3′ end of β_2 was cloned as an *Eco*RI-*Bgl*II fragment in the pRc/CMV vector (containing a neomycin resistance gene; Invitrogen, San Diego, CA). Within this sequence is a unique *Apa*I site at position 1980. The C-terminal end was rebuilt from this site with 10 overlapping oligonucleotides and amplification by the polymerase chain reaction to obtain the appropriate hybrids. For the β_2/β_1 chimeric protein,

amino acid 752 of β_1 (cytoplasmic domain) was joined to amino acid 723 of β_2 and for the β_2/β_7 chimeric protein, amino acid 747 of β_7 (cytoplasmic domain) was joined to amino acid 723 of β_2. The deletion mutant of LFA-1 was made by truncation of the β_2 cytoplasmic tail from amino acid 724 (see Figure 2).

Cell Culture and Transfection

Stable LFA-1-expressing K562 transfectants were established by electroporation of 10^7 cells in 0.8 ml of phosphate-buffered saline at 280 V and 960 μF with the α_L (in pCDM8) and the wild-type β_2 subunit (in pRc/CMV), the α_L and β_2 cytoplasmic truncated subunit (Δ724), or the α_L and the chimeric β_2/β_1- or β_2/β_7-chain of LFA-1. K562-LFA-1 transfectants were cultured in RPMI 1640 medium (Life Technologies, Paisley, Scotland), supplemented with 10% fetal calf serum (BioWhittaker, Verviers, Belgium), and 1% antibiotics/antimycotics (Life Technologies). After 48 h the neomycin analogue, Geneticin (2 mg/ml, Life Technologies) was added to the culture medium. The different transfectants were sorted three times to obtain a homogenous population of cells expressing high levels of LFA-1. Positive cells were stained directly with fluorescein isothiocyanate (FITC)-labeled TS2/4 mAb. Cells were sorted with the Coulter Epics Elite (Coulter, Hialeah, FL).

Immunofluorescence Analysis

Expression of LFA-1 on the transfectants was determined by immunofluorescence. Cells (2×10^5 cells) were incubated (30 min, 4°C) in phosphate-buffered saline, containing 0.5% (wt/vol) bovine serum albumin (BSA, Boehringer Mannheim, Mannheim, Germany) and 0.01% sodium azide (10 mM, Merck, Hohenbrunn, Germany), with appropriate dilutions of either an anti-integrin mAb or an isotype-matched control antibody, followed by incubation with FITC-labeled goat $(Fab')_2$ anti-mouse IgG mAb (Zymed Laboratories, San Francisco, CA) for 30 min at 4°C. The relative fluorescence intensity was measured by FACScan analysis (Becton Dickinson, Oxnard, CA).

Adhesion Assay

Binding of LFA-1-positive cells to ICAM-1 was performed with ICAM-1 fusion proteins consisting of the five Ig-like domains of ICAM-1 fused to a human IgG1 Fc fragment (ICAM-1Fc). ICAM-1Fc was generated by transfecting mouse L cells with the vector pICAM-1-IgG1 by calcium phosphate precipitation (calcium phosphate transfection system, Life Technologies) using a standard protocol (Fawcett *et al.*, 1992). Culture supernatant was purified by protein A chromatography and eluted by 3.5 M $MgCl_2$ and 10% glycerol.

Ninety-six-well flat-bottomed plates (MaxiSorp, Nunc, Roskilde, Denmark) precoated with 50 μl of goat anti-human Fc-specific $F(ab')_2$ (4 μg/ml; Jackson ImmunoResearch Laboratories, Westgrove, PA) for 1 h 37°C and blocked with 1% BSA in TSM (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) for 30 min at 37°C were coated with 0.2 μg/ml ICAM-1Fc protein overnight at 4°C. Stably transfected CD11a/CD18 K562 cells were labeled with $Na_2{}^{51}CrO_4$ (Amersham International, Buckinghamshire, England) for 45 min at 37°C. Radiolabeled cells were washed and preincubated for 15 min at room temperature with different stimuli [50 nM phorbol 12-myristate 13-acetate (PMA, Calbiochem, La Jolla, CA), 5 μg/ml KIM185, or 5 μg/ml TS2/16] and/or blocking mAbs (10 μg/ml). Cells were allowed to adhere for 45 to 60 min at 37°C. Unbound cells were removed by washing with TSM supplemented with 0.5% (wt/vol) BSA. The adherent cells were lysed with 100 μl of 2% Triton X-100 and radioactivity was quantified in a gamma counter. Results are expressed as the mean percentage of cells binding from triplicate wells. Values are depicted as integrin-specific adhesion: percentage of cells binding − percentage of cells binding in the presence of an integrin-blocking mAb (NKI-L15 or SAM-1).

Radiolabeling and Immunoprecipitation

Transfectants were surface labeled with $Na^{125}I$ (Amersham International). For immunoprecipitation, 10^7 cells were solubilized for 1 h at 4°C in immunoprecipitation buffer (IPB), which contained 1% Nonidet P-40 (Sigma, St. Louis, MO), 50 mM triethanolamine (pH 7.8, Sigma), 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, and as protease inhibitors (Sigma) 1 mM phenylmethylsulfonyl fluoride, 0.02 mg/ml ovomucoid trypsin inhibitor, 0.02 mg/ml leupeptin, and 1 mM Nα-P-tosyl-L-lysine chloromethyl ketone were added. Nuclear debris was removed from the lysates by centrifugation at 13,000 × *g* for 15 min at 4°C. Lysates were precleared by successive incubation with mouse IgG covalently coupled to protein A-Sepharose CL-4B beads (Pharmacia, Piscataway, NJ). Precleared cell lysates were immunoprecipitated with specific mAb, directed against either LFA-1 or VLA-5, coupled to protein A-Sepharose CL-4B for 1 to 2 h at 4°C. The immunoprecipitates were removed from the lysates by centrifugation at 13,000 × *g*. Subsequently, immunoprecipitates were washed extensively in IPB and analyzed under reducing conditions with 5% β-mercaptoethanol in SDS sample buffer. SDS-PAGE was carried out on vertical slab gels (5–15%) according to a modification of the Laemmli procedure (Laemmli, 1970). Kodak XAR film was used in combination with intensifier screens (Cronex Lightning Plus; DuPont, Newton, CT) for autoradiography of ^{125}I-labeled materials.

Confocal Microscopy

Cells were fixed with 0.5% paraformaldehyde. Fixed cells were stained with TS2/4 mAb (10 μg/ml) for 30 min at 37°C followed by incubation with FITC-labeled goat $(Fab')_2$ anti-mouse IgG mAb (Zymed Laboratories) 30 min at room temperature. Cells were attached to poly-L-lysine–coated glass slides, after which cell surface distribution of integrins was determined by confocal laser scanning microscopy (CLSM) at 488 nm with a krypton/argon laser (Bio-Rad 1000, Hercules, CA). The CLSM settings were: lens, 60×; gain, 1300; pinhole, 1.5 μm; and magnification, 1.5×. The same instrument settings of the CLSM were used throughout the distinct experiments.

RESULTS

Expression and Function of Wild-Type LFA-1 in the Erythroleukemic K562 Cells

LFA-1 ($\alpha_L\beta_2$) is an adhesion receptor that is exclusively expressed on lymphocytes. To investigate the importance of a lymphocytic environment for the adhesive function of LFA-1, we transfected both wild-type α_L- and β_2-chain cDNA in erythroleukemic K562 cells that do not express the lymphocyte-specific β_2 and β_7 integrins endogenously. The transfectants express high levels of the heterodimer LFA-1 on their cell surface as detected by staining of the cells with mAb directed against either α_L (SPV-L7) or β_2 (60.3; see Figure 3A).

The capacity of LFA-1 on K562 cells to bind its ligand ICAM-1 was determined after activation of LFA-1 with PMA or the activating anti-β_2 mAb (KIM185) and was compared with the adhesion capacity of VLA-5, the only endogenously expressed β_1 integrin. Figure 1A demonstrates that the LFA-1-trans-

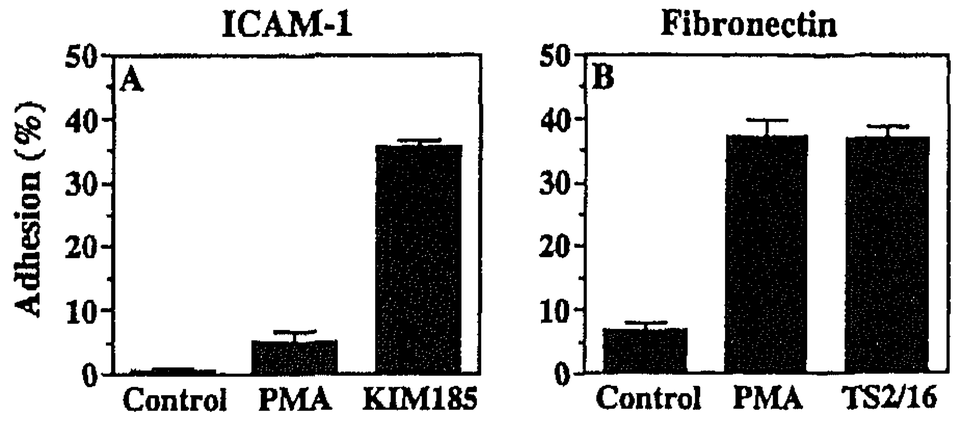

Figure 1. Capacity of K562 $\alpha_L\beta_2$ transfectants to bind to ICAM-1 or fibronectin. K562-$\alpha_L\beta_2$ cells were preincubated in medium (control), PMA (50 nM), the activating anti-β_2 mAb KIM185, or the activating anti-β_1 mAb TS2/16 (5 μg/ml). Depicted is the mean percentage of either LFA-1-specific adhesion to ICAM-1 or VLA-5-specific adhesion to fibronectin. Integrin-specific adhesion: percentage of cells binding − percentage of cells binding in the presence of an integrin blocking mAb (NKI-L15 or SAM-1). Data are representative of four experiments.

fected K562 cells express functional LFA-1 molecules because KIM185 readily induces LFA-1-mediated ICAM-1 binding. Surprisingly, PMA was not able to induce LFA-1-mediated adhesion to ICAM-1. This is not due to a general nonresponsiveness of K562 cells to PMA because it significantly enhances VLA-5-mediated binding to fibronectin. Similar to KIM185, the activating anti-β_1 mAb TS2/16 effectively stimulated VLA-5-mediated adhesion (Figure 1B).

The observation that PMA could enhance β_1 integrin (VLA-5)-mediated adhesion but not β_2 integrin-(LFA-1) mediated adhesion suggests that distinct signaling elements are involved in the PMA-induced β_2 and β_1 integrin activation.

Expression of β_2-Chimeric LFA-1 Molecules in Erythroleukemic K562 Cells

To determine whether the observed differences between PMA responsiveness of β_1 and β_2 integrins are due to differences in the cytoplasmic tail, we generated LFA-1 molecules in which the cytoplasmic tail of the β_2-chain was truncated close to the transmembrane region at amino acid position 724 (K562-$\alpha_L\beta_2$/Δ724) or replaced with that of the β_1-chain or the β_7-chain (K562-$\alpha_L\beta_2/\beta_1$ and K562-$\alpha_L\beta_2/\beta_7$, respectively). Figure 2 shows the amino acid sequence of the different LFA-1 chimeras. Asterisks mark the amino acid sequence homology of the cytoplasmic domain of the three distinct β-chains. Both β_2 and β_7 integrins are predominantly expressed by lymphocytes, whereas β_1 integrins are widely distributed.

The chimeric β_2-chains: β_2/β_1, β_2/β_7 and β_2/Δ724 were transfected along with the wild-type α-chain (α_L) in K562. All transfectants expressed equally high levels of LFA-1 (Figure 3A). Our unpublished observations demonstrated that LFA-1 was expressed as a heterodimer on the cell surface, since all transfectants expressed equally high levels of the MHM23 epitope that has been reported to detect an α/β-association-dependent epitope on LFA-1 (Hildreth and August, 1985). These data demonstrate that deletion of the β_2 cytoplasmic tail or replacement of the β_2 cytoplasmic tail for the β_1 or β_7 cytoplasmic tail does not alter the overall conformation of the LFA-1 α/β heterodimer. All transfectants show similar levels of the endogenously expressed β_1 integrin, VLA-5 (Figure 3A).

To verify that the chimeric LFA-1 molecules $\alpha_L\beta_2/\beta_1$ and $\alpha_L\beta_2/\beta_7$ did not associate with endogenous α_5-chain in K562, LFA-1 and VLA-5 were immunoprecipitated from all transfectants. β_2 wild-type, chimeric β_2-chains, and $\alpha_L\beta_2/\Delta$724 associated with α_L and not with α_5 (Figure 3B, lanes C, D, F, G, I, J, L, and M, observed as the thick bands of 185 and 95 kDa). Similarly, immunoprecipitation with an anti-α_5 mAb showed that VLA-5 does not associate with α_L or β_2 (Figure 3B, lanes A, E, H, and K, observed as one thick band of 130–135 kDa, because, under reduced conditions, the α_5- and β_1-chains have approximately the same molecular weight).

Cytoplasmic β_1 Domain Restores PMA Responsiveness of LFA-1 in K562 Cells

Next, we investigated the capacity of the cytoplasmic tail of the β_1 or β_7 integrin to restore activation of LFA-1 by PMA. Wild-type LFA-1 (Figure 4) and the chimeric LFA-1 transfectants $\alpha_L\beta_2/\beta_1$ and $\alpha_L\beta_2/\beta_7$ showed increased binding when activated by the LFA-1-activating antibody KIM185. The cytoplasmic tail of β_7 in the $\alpha_L\beta_2/\beta_7$ transfectant did not restore PMA-

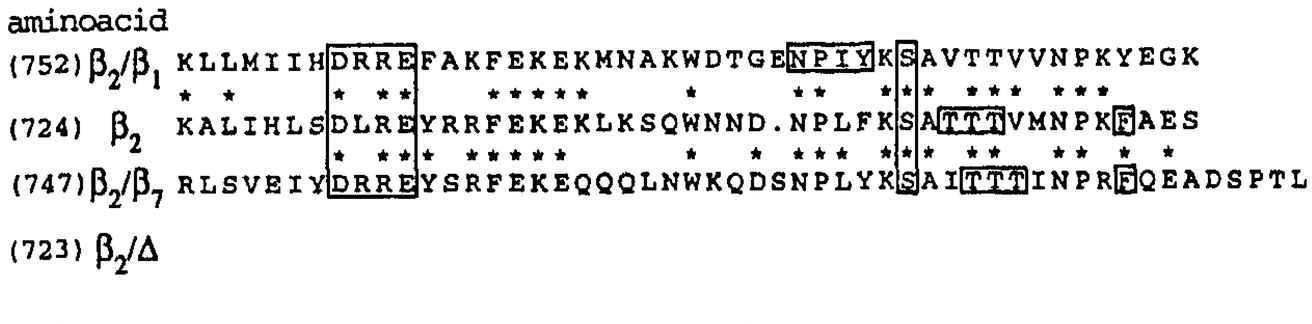

Figure 2. Comparison of amino acid sequence of the β_1, β_7, and β_2 cytoplasmic domain. The amino acids sequence is depicted in the single-letter code. One amino acid gap was inserted into the β_2-chain for alignment with the other β-chains. Asterisks mark the homology in amino acid sequences between either the β_7 and β_2 or the β_1 and β_2 cytoplasmic domain. The boxes indicate amino acid sequences implicated in integrin function and activation. Between brackets is depicted the amino acid position of β_2, at which the cytoplasmic domain of the β_2 is deleted or the first amino acid sequence of the corresponding β_1 or β_7 cytoplasmic domain is joined to the transmembrane part of β_2, creating the distinct chimeric LFA-1 transfectants (Erle *et al.*, 1991).

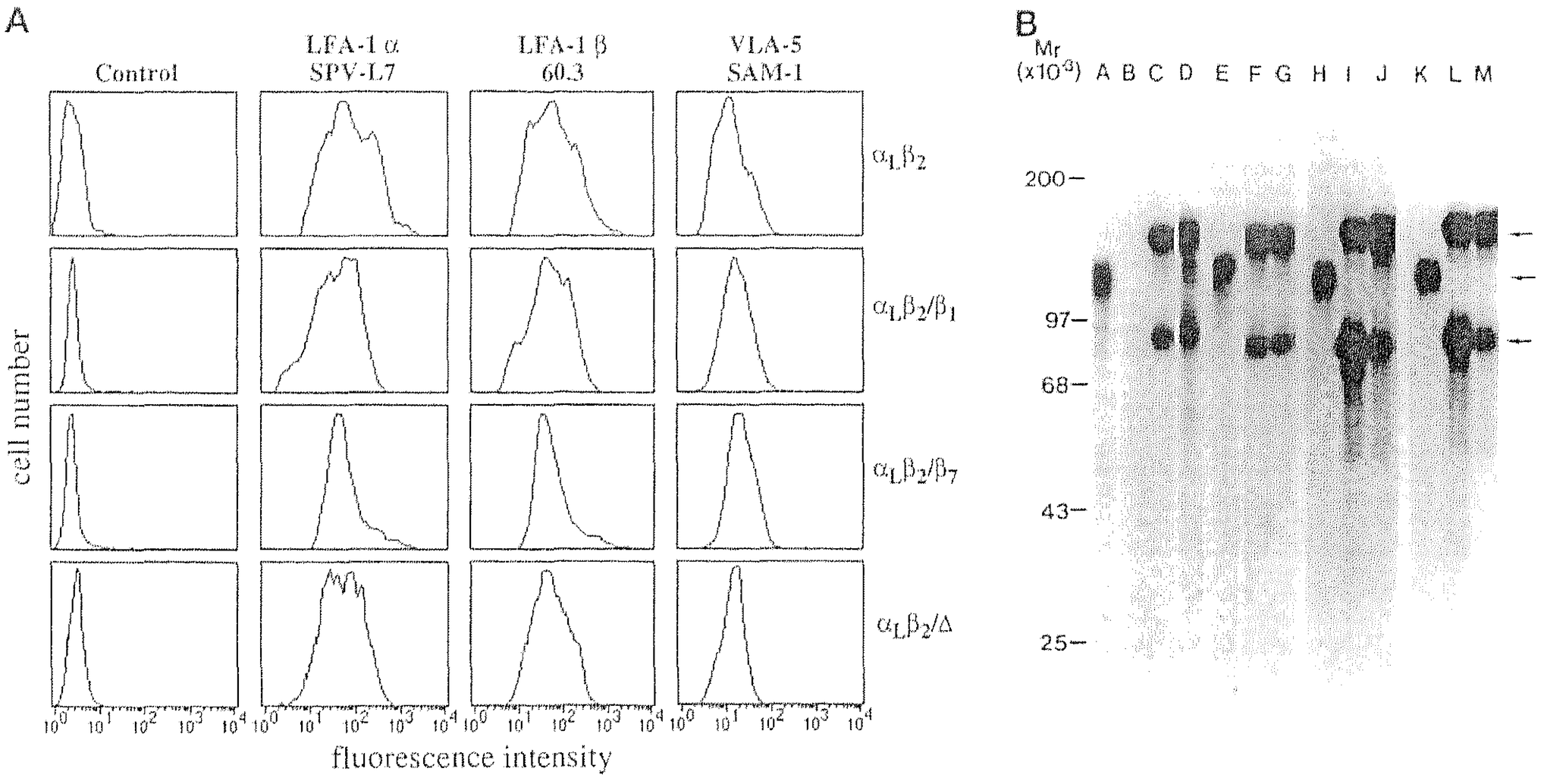

Figure 3. Expression and immunoprecipitation of LFA-1 and VLA-5 on the chimeric LFA-1 transfectants. (A) K562-$\alpha_L\beta_2$, K562-$\alpha_L\beta_2/\beta_1$, K562-$\alpha_L\beta_2/\beta_7$, and $\alpha_L\beta_2/\Delta724$ transfectants were stained with isotype-matched control antibodies or specific antibodies directed against the α subunit (SPV-L7) or the β subunit (60.3) of LFA-1 or against VLA-5 (SAM-1). (B) K562-$\alpha_L\beta_2$ transfectants (lanes A–D), K562-$\alpha_L\beta_2/\Delta724$ (lanes E–G), K562-$\alpha_L\beta_2/\beta_7$ (lanes H and J), and K562-$\alpha_L\beta_2/\beta_1$ (lanes K–M) were immunoprecipitated with the anti-VLA-5 mAb SAM-1 (lanes A, E, H, and K), the anti-LFA-1α mAb (SPV-L7) (lanes C, F, I, and L), or the anti-LFA-1β mAb 60.3 (lanes D, G, J, and M). As a control for specifically precipitated proteins, lysates were also precipitated with normal mouse serum (lane B).

induced binding to ICAM-1. In contrast, the cytoplasmic tail of β_1 in the chimeric $\alpha_L\beta_2/\beta_1$ transfectant restored PMA responsiveness, which is not dependent on expression levels of chimeric $\alpha_L\beta_2/\beta_1$, since our unpublished results have shown that transfectants expressing low levels of β_2/β_1 also respond to PMA. In addition, increasing the concentration of coated ICAM-1Fc did not alter the PMA nonresponsiveness of wild-type LFA-1 in K562 (Figure 5A). Whereas PMA stimulated binding of chimeric $\alpha_L\beta_2/\beta_1$ to ICAM-1 to an extent similar to the KIM185-stimulated adhesion of these cells, both independent of the concentration of coated ICAM-1Fc (Figure 5B). Similarly, also titration using higher doses of PMA did not alter the PMA nonresponsiveness of wild-type LFA-1 in K562 (Figure 5C). In contrast to both the chimeric and wild-type LFA-1 transfectants, the $\alpha_L\beta_2/\Delta724$ transfectant binds ICAM-1 equally well in the absence or presence of the activating mAb KIM185 or PMA (Figure 4). Adhesion was LFA-1 mediated since antibodies directed against LFA-1 (Figure 4) or ICAM-1 inhibited the cell binding completely and mock transfectants always showed less than 2% adhesion to ICAM-1 as demonstrated by our unpublished results. These data indicate that the β_1, but not the β_7, cytoplasmic domain is capable of restoring PMA responsiveness of LFA-1 in K562 and that the cytoplasmic tail of β_2 is important in regulating LFA-1 activation. The observation that PMA could still enhance β_1 integrin (VLA-5)-mediated adhesion to fibronectin of all transfectants (Figures 1 and 4) again suggests that distinct intracellular routes are involved in β_2 and β_7 compared with β_1 integrin activation.

Clustering of LFA-1 on the Cell Surface Is Regulated by the β-Chain

We determined whether truncation of the β_2 cytoplasmic domain or replacement of the β_2 cytoplasmic domain by the corresponding β_1 or β_7 cytoplasmic domains affected the distribution of $\alpha_L\beta_2$ integrins at the cell surface. Altered distribution of integrins may affect the avidity state of the receptors, facilitating ligand binding (Figdor *et al.*, 1990; van Kooyk *et al.*, 1994; Lub *et al.*, 1995). Therefore, wild-type ($\alpha_L\beta_2$), the deletion mutant ($\alpha_L\beta_2/\Delta724$), and the β_2-chimeric LFA-1 transfectants ($\alpha_L\beta_2/\beta_1$ and $\alpha_L\beta_2/\beta_1$) were stained with the anti-LFA-1 antibody NKI-L16, which detects Ca^{2+}-dependent clustering of LFA-1 on the cell surface (Keizer *et al.*, 1988; van Kooyk *et al.*, 1994). Figure 6 demonstrates that wild-type LFA-1 transfectants express low levels of the L16 epitope compared

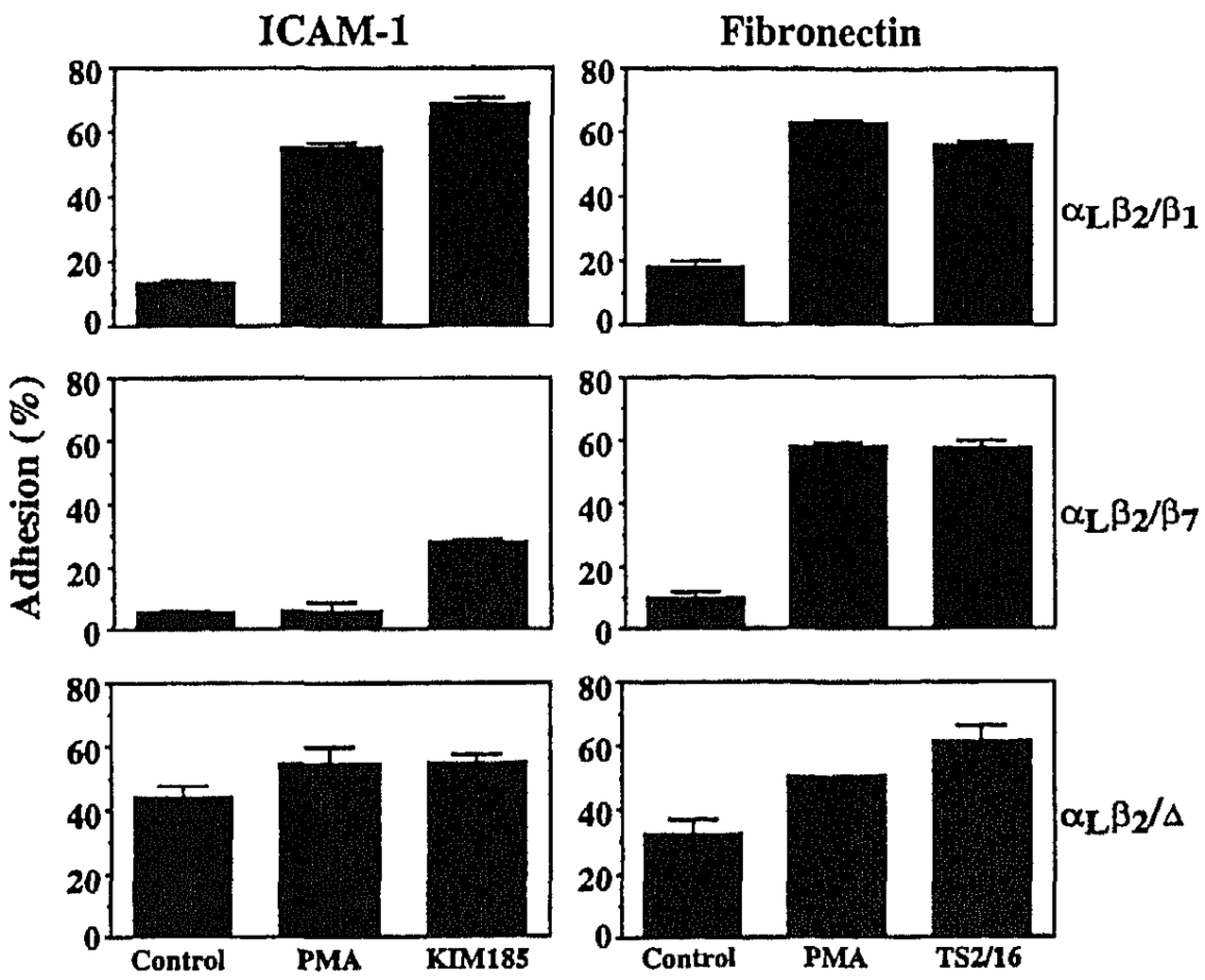

Figure 4. Cytoplasmic β_1 domain restores PMA responsiveness of LFA-1 in K562 cells. K562-$\alpha_L\beta_2/\beta_1$ and K562-$\alpha_L\beta_2/\beta_7$ transfectants and K562-$\alpha_L\beta_2/\Delta724$ were preincubated in medium (control), PMA (50 nM), or with the activating anti-β_2 mAb KIM185 (5 μg/ml) or activating anti-β_1 mAb TS2/16 (5 μg/ml) and allowed to adhere for 45–60 min at 37°C. Adhesion was performed in the absence or presence of the LFA-1 blocking mAb (NKI-L15) or VLA-5 blocking mAb (Sam-1) to ICAM-1 or fibronectin, respectively. Depicted is the mean percentage of LFA-1-specific binding to ICAM-1 and VLA-5-specific adhesion to fibronectin of three independent wells. Integrin-specific adhesion: Percentage of cells binding − percentage of cells binding in the presence of an integrin blocking mAb. Data are representative of three experiments.

with expression of a regular anti-LFA-1 (NKI-L16: SPV-L7 peak channel ratio is approximately 0.3). Similarly, $\alpha_L\beta_2/\beta_7$ transfectants show low L16 expression indicating that the cytoplasmic domain of β_7 does not affect LFA-1 distribution (NKI-L16:SPV-L7 peak channel ratio is approximately 0.3). In marked contrast, $\alpha_L\beta_2/\beta_1$ and $\alpha_L\beta_2/\Delta724$ transfectants show high expression of the L16 epitope, comparable to expression of the SPV-L7 epitope, indicating that all LFA-1 molecules express the L16 epitope (NKI-L16:SPV-L7 peak channel ratio is roughly 1).

To determine whether deletion or replacement of the β_2 cytoplasmic domain for the β_1 cytoplasmic domain directly affects the distribution of LFA-1 on the cell surface, CLSM studies were performed with all LFA-1 transfectants. Figure 7 demonstrates that LFA-1 is homogenously distributed on the cell surface of both wild-type (Figure 7A, only two of seven cells show some clustering of LFA-1) and chimeric $\alpha_L\beta_2/\beta_7$ transfectants (Figure 7C), whereas on the cell surface of all chimeric $\alpha_L\beta_2/\beta_1$ (Figure 7B) and $\alpha_L\beta_2/\Delta724$ transfected cells (Figure 7D), LFA-1 is distributed in clusters. Clustering was not attributed to a higher expression level of LFA-1 (Figure 3A). These data indicate that either deletion or replacement of the β_2 cytoplasmic domain for the β_1 cytoplasmic domain dramatically affects the distribution of LFA-1, as well as results in an increment in the L16 epitope expression. Moreover, our unpublished results demonstrated that VLA-5 is similarly distributed into small clusters on all K562 transfectants. It should be noted that despite the strong clustering of $\alpha_L\beta_2/\beta_1$ receptors on the cell surface stable binding to ICAM-1 still depends on activation of the receptor with PMA, in contrast to the $\alpha_L\beta_2/\Delta724$ transfectants that express constitutively active LFA-1 molecules.

DISCUSSION

Our results demonstrate that 1) PMA cannot activate LFA-1 when expressed in erythroleukemic K562 cells, despite the fact that PMA readily activates endogenously expressed β_1 integrins. 2) Expression of β_2 chimeric receptors in K562 reveals that the β_1 cytoplasmic tail but not the β_7 cytoplasmic tail restores PMA responsiveness of LFA-1. 3) Lymphocyte-specific signal transduction elements may be involved in β_2 and β_7 integrin activation that are absent in K562 cells. 4) Inside-out signaling (by PMA) is mediated by the β-chain of integrins. 5) Replacement of the β_2 cytoplasmic tail with that of β_1 alters the surface distribution of LFA-1 into clusters and facilitates ICAM-1 binding. 6) Deletion of the entire β_2 cytoplasmic domain localizes LFA-1 molecules into clusters and results in constitutively active receptors.

The PMA nonresponsiveness of LFA-1 in K562 cells is not restricted to this integrin only but holds also for the other two β_2 integrins Mac-1 and p150.95 (Ortlepp *et al.*, 1995) and is not dependent on the concentration of the ligand presented or on the concentration of the stimulus (Figure 5). Our finding that the cytoplasmic tail of the β_7 integrin cannot revert the PMA nonresponsiveness of LFA-1 in these cells predicts that transfection of β_7 integrins into K562 (these cells do

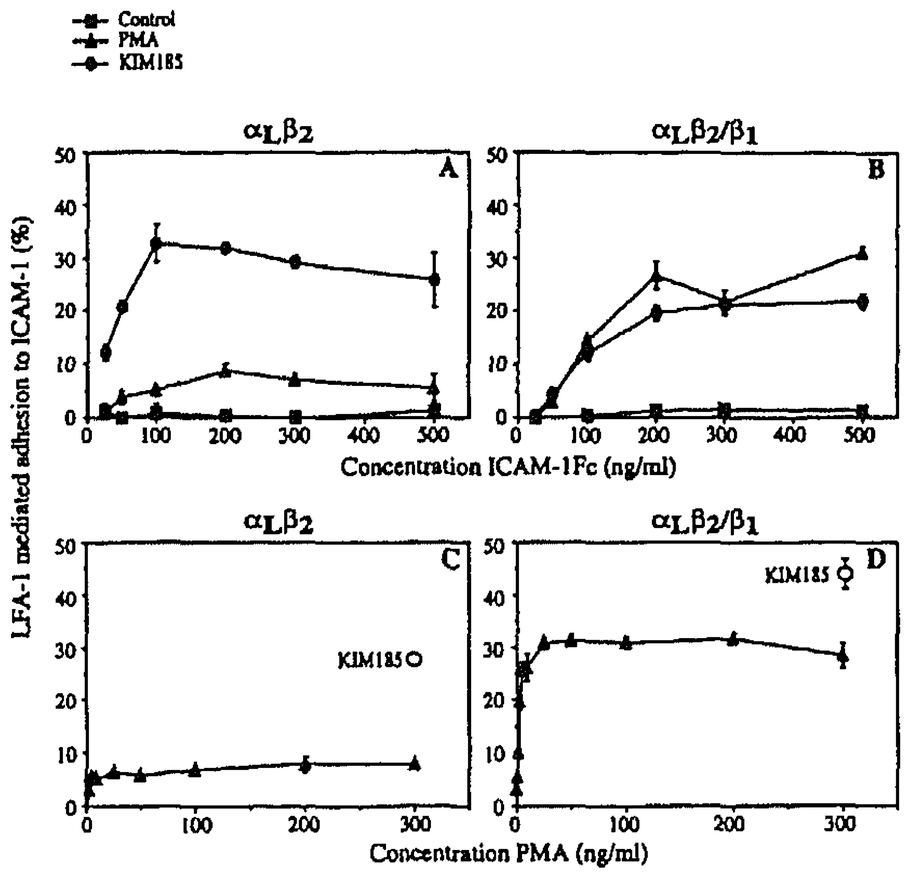

Figure 5. PMA nonresponsiveness of LFA-1 expressed in K562 is independent on the concentration ICAM-1 coated (A and B) or the amount of stimulus PMA to induce adhesion (C and D). K562-$\alpha_L\beta_2$ (A) and K562-$\alpha_L\beta_2/\beta_1$ (B) were preincubated in medium (■), PMA (50 nM, ▲), or with the activating anti-β_2 mAb KIM185 (5 μg/ml, ●) and allowed to adhere to different concentrations of ICAM-1Fc (500–25 ng/ml) for 45 to 60 min at 37°C in the absence or presence of the LFA-1-blocking mAb (NKI-L15). Similarly, K562-$\alpha_L\beta_2$ (C) and K562-$\alpha_L\beta_2/\beta_1$ (D) were stimulated with different concentrations of PMA (5–300 ng/ml, ▲). High amounts of PMA were still incapable of restoring the PMA nonresponsiveness of LFA-1, whereas the activating mAb KIM185 enhanced adhesion (○). Depicted is the mean percentage of LFA-1-specific binding to ICAM-1 of three independent wells. LFA-1-specific adhesion: percentage of cells binding − percentage of cells binding in the presence of a LFA-1-blocking mAb (NKI-L15). Data are representative of three experiments.

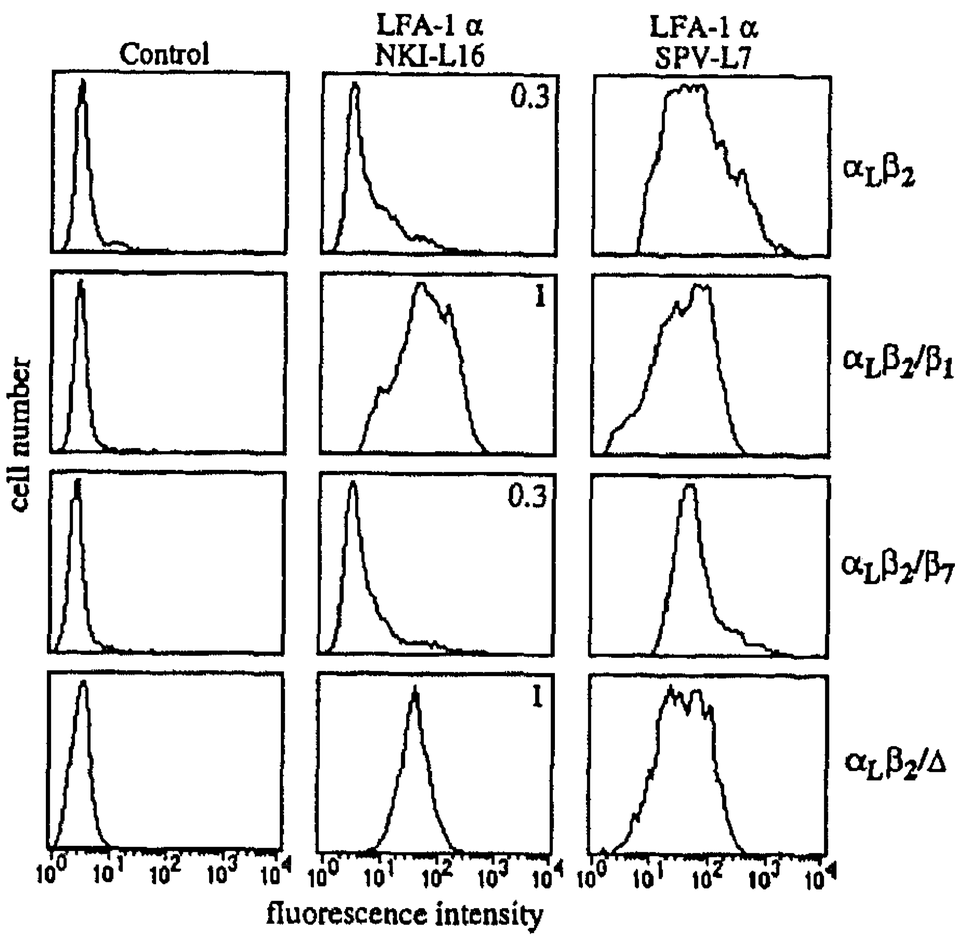

Figure 6. Expression of the LFA-1-clustering–dependent L16 epitope. K562-$\alpha_L\beta_2$, K562-$\alpha_L\beta_2/\beta_1$, K562-$\alpha_L\beta_2/\beta_7$, and K562-$\alpha_L\beta_2$/Δ724 transfectants were stained with isotype-matched control antibodies, the mAb NKI-L16, which reports LFA-1 clustering, or with a regular mAb directed against the α subunit (SPV-L7) of LFA-1. Data are representative of five experiments.

not endogenously express β_7 integrins), such as $\alpha_4\beta_7$, will not allow the cells to respond to PMA. This suggests that K562 cells lack intracellular signaling elements required to regulate adhesion through the lymphocyte-specific β_2 and β_7 integrins. The finding that the cytoplasmic tail of the β_1 integrin completely restores PMA responsiveness of LFA-1 in K562 cells cannot be attributed to a higher expression level of the β_2/β_1 chimeric receptors, since our unpublished results demonstrate that transfectants expressing low levels of β_2/β_1 also respond to PMA. Our finding that the β_1 integrin VLA-5, which is endogenously expressed by the K562 cells, responds to PMA by allowing the cells to bind its ligand fibronectin demonstrates that the intracellular signaling elements activated by PMA are different for β_1 integrins compared with β_2 and β_7 integrins.

Our results demonstrate that the cytoplasmic domain of the β-chain of integrins plays an important role in the inside-out signaling initiated by PMA. Whether PMA directly or indirectly activates β_2 integrins via its β-chain requires further study. Previously, it has been shown that PMA leads to phosphorylation of the serine residue at position 756 in the β_2 cytoplasmic domain (Hibbs *et al.*, 1991a). However, mutation of this serine residue does not impair ICAM-1 binding, demonstrating that phosphorylation at this position is not crucial for adhesion to ICAM-1 (Hibbs *et al.*, 1991a). Alignment of the β_1 and β_7 cytoplasmic domains to the β_2 cytoplasmic domain revealed that the serine residue is conserved in both β_1 and β_7 (Figure 2), indicating that this serine residue cannot explain the differences observed in the chimeric receptors expressed in K562. Mutations of a triplet of threonines (amino acids 758–760) and a phenylalanine residue at position 766 in the β_2 cytoplasmic domain have been shown to completely abrogate ICAM-1 adhesion. Interestingly, a similar triplet of threonines and phenylalanine residue are present in the β_7 cytoplasmic domain and are absent in the β_1 cytoplasmic domain. Therefore, it is tempting to speculate that these residues may be important in the lymphocyte-specific signal transduction pathway (Hibbs *et al.*, 1991a). To determine the precise residues in the β_1 cytoplasmic domain involved in the PMA induced signaling, mutations studies should be performed (work in progress).

Our LFA-1 deletion mutant K562-$\alpha_L\beta_2$/Δ724, which lacks the complete β_2 cytoplasmic tail (45 amino acids long), was found to be constitutively active in K562

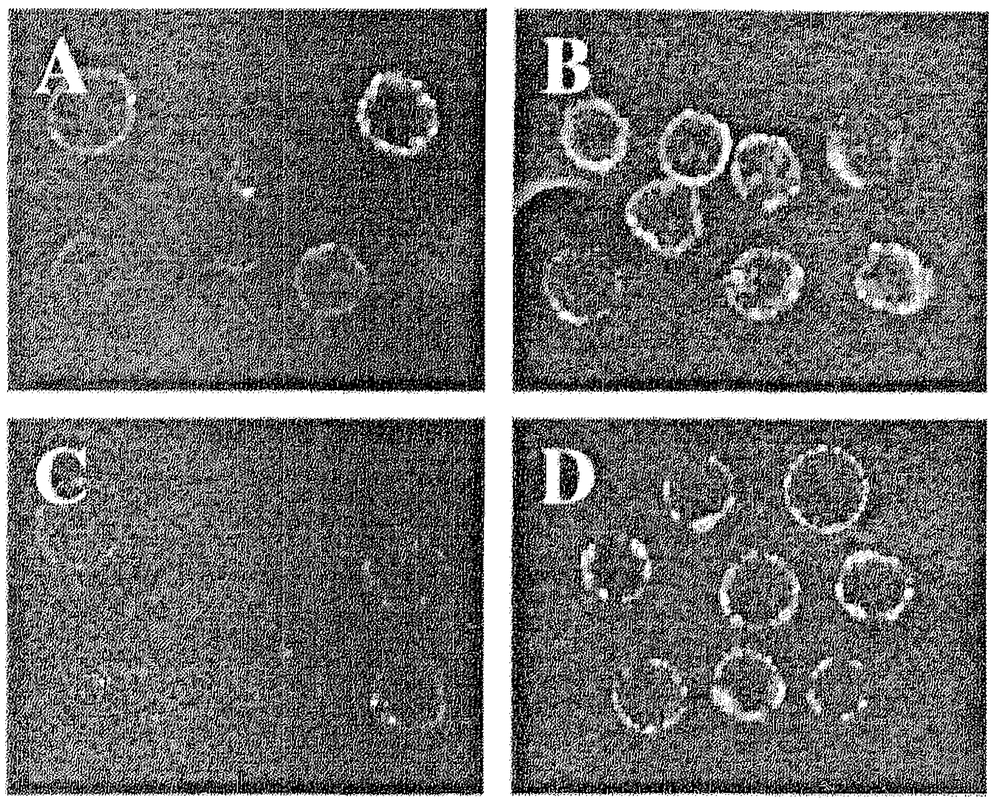

Figure 7. Surface distribution of LFA-1 as determined by CLSM. Cells were fixed (0.5% paraformaldehyde) and subsequently stained with the anti-LFA-1 mAb TS2/4 and goat anti-mouse $(Fab')_2$-FITC second antibodies. The difference in colors depicts the intensity of expression of LFA-1 molecules on the cell surface from red to yellow to green equals low amounts to high amounts. LFA-1 is localized in large clusters on K562-$\alpha_L\beta_2/\beta_1$ (B) and K562-$\alpha_L\beta_2/\Delta724$ (D), whereas LFA-1 is more homogenously expressed on K562-$\alpha_L\beta_2$ (A) and K562-$\alpha_L\beta_2/\beta_7$ transfectants (C). The instrument settings of the CLSM were the same for all micrographs: lens, 60×; gain, 1300; pinhole, 1.5 μm; magnification, 1.5×. One of three experiments is shown.

cells. Similar observations were made when the complete cytoplasmic tail of the β_7 was deleted ($\Delta747$, deletion of 51 amino acids) in $\alpha_4\beta_7$ (Crowe *et al.*, 1994) or when the complete cytoplasmic tail of the β_3 was deleted ($\Delta744$, deletion of 45 amino acids, the β_3 cytoplasmic tail is 47 amino acids long) in $\alpha_{IIb}\beta_3$ (Hughes *et al.*, 1995). In contrast, when the first 28 amino acids of the N-terminal region of the cytoplasmic tail of β_2 were deleted, no effect on ICAM-1 binding activity was observed (Hibbs *et al.*, 1991a). Similarly, mutants containing deletion greater than 5–15 amino acids at the C-terminal end of the β_1 cytoplasmic domain neither promoted adhesion nor localization of β_1 integrins in focal contacts (Solowska *et al.*, 1989; Hayashi *et al.*, 1990; Marcantonio *et al.*, 1990). In contrast, deletion of 39 amino acids C-terminal of the β_1 cytoplasmic tail ($\Delta759$, β_1 cytoplasmic tail is 46 amino acids long) impairs the binding capacity to both laminin and fibronectin. Likewise, partial removal of the C-terminal part of the β_7 cytoplasmic domain ($\Delta773$) of the $\alpha_4\beta_7$ integrin displayed no ligand binding activity to VCAM-1 (Hayashi *et al.*, 1990; Crowe *et al.*, 1994). These distinct findings can be attributed to the sites where the cytoplasmic tails of the β-chains were deleted. The different β cytoplasmic tails share high homology in their membrane-proximal region, especially the DRRE sequence is conserved between the different β cytoplasmic tails (Figure 3, $D^{759}RRE^{762}$ of β_1). Comparison of the positions at which the distinct β cytoplasmic tail were truncated suggests that deletion of the conserved aspartic acid residue corresponding to position 731 in the β_2 tail results in a constitutively active molecule, indicating that this residue is most important in regulating integrin activation. In contrast, it seems that in deletion mutants in which this conserved aspartic acid residue is not removed, the integrin activity can still be regulated. Moreover, amino acid sequences more C-terminal of the β cytoplasmic tail are more likely important in ligand binding capacity rather than in regulation of integrin activation (Solowska *et al.*, 1989; Hayashi *et al.*, 1990; Marcantonio *et al.*, 1990; Hibbs *et al.*, 1991a,b; Crowe *et al.*, 1994; Hughes *et al.*, 1995). The hypothesis that the conserved aspartic acid residue (Figure 3, D^{759} of β_1) is pivotal in the regulation of integrin activation, is also supported by our finding that the LFA-1 deletion mutant K562-$\alpha_L\beta_2/\Delta724$, which lacks the entire cytoplasmic β_2 tail including the conserved DRRE sequence, has shown to be constitutively active.

The observation that β_2/β_1 chimeric LFA-1 receptors localize in clusters on the cell membrane but the β_2/β_7 chimeric LFA-1 and wild-type LFA-1 do not demonstrates that the β_1 cytoplasmic domain plays an important role in mobilizing LFA-1 into clusters. Others have demonstrated that the NPIY motif within the β_1 cytoplasmic domain is important for localization of the integrin into focal contacts (Reszka *et al.*, 1992; Mauro and Dixon, 1994; O'Toole *et al.*, 1995). Interestingly, this motif is absent in the β_2 and β_7 cytoplasmic domain (Figure 2), which may explain the absence of a clustered LFA-1 distribution on the wild-type $\alpha_L\beta_2$ and the chimeric $\alpha_L\beta_2/\beta_7$ transfectants. This finding again demonstrates that clustering of LFA-1 in itself is essential but not sufficient to stimulate stable LFA-1/ICAM-1 adhesion. Both high-avidity (clustering) and high-affinity (active conformation induced by PMA) states of LFA-1 cooperated for strong adhesion.

It has been demonstrated that integrins can associate with cytoskeletal components (α-actinin and talin), particularly through the β-chain, and thereby regulate the cell surface distribution of the integrin (Burn *et al.*, 1988; Pavalko and LaRoche, 1993). Deletion of the cytoplasmic tail may disconnect the integrin from the cytoskeleton and allow lateral movement of the integrin at the cell membrane, explaining the clustered distribution of LFA-1 on the $\alpha_L\beta_2/\Delta724$ transfectants. Furthermore, it may well be that clustering of integrins on the cell surface colocalizes important kinases essential for proper signal transduction (Miyamoto *et al.*, 1995). Not only is the intracellular conformation or association with regulatory proteins affected by clustering of integrins on the cell surface but also the extracellular conformation is altered, as evidenced by enhanced L16 epitope expression when the β_2 cyto-

plasmic domain was deleted or replaced for the β_1 cytoplasmic domain. This may be attributed to distinct interactions with cytoplasmic proteins that affect the extracellular conformations of the integrin molecule.

We have demonstrated that the cytoplasmic domain of the β-chain of integrins is responsible for the cell surface distribution of the integrin, regulating the activation of the integrin and that it plays an essential role, either directly or indirectly, in PMA-induced signaling. Furthermore, PMA can activate β_1 integrins on K562, whereas it failed to activate the lymphocyte-specific β_2 and β_7 integrins in K562, suggesting that PMA activates β_2 and β_7 integrins through lymphocyte-specific elements that are absent in the nonlymphocytic cell line K562.

ACKNOWLEDGMENTS

We thank Dr. E. Martz and Dr. J.M. Harlan for kindly providing antibodies and Dr. D. Simmons for providing the vector containing the ICAM-1/IgG fusion construct. We thank Dr. A. Sonnenberg for providing the K562 cells. This work was supported by the Netherlands Organization for Scientific Research (grants 900–512-143 and 900–509-185).

REFERENCES

Altevogt, P., Hubbe, M., Ruppert, M., Lohr, J., von Hoegen, P., Sammar, M., Andrew, D.P., McEvoy, L., Humphries, M.J., and Butcher, E.C. (1995). The alpha 4 integrin chain is a ligand for alpha 4 beta 7 and alpha 4 beta 1. J. Exp. Med. *182*, 345–355.

Altieri, D.C. (1991). Occupancy of CD11b/CD18 (Mac-1) divalent ion binding site(s) induces leukocyte adhesion. J. Immunol. *147*, 1891–1898.

Andrew, D., Shock, A., Ball, E., Ortlepp, S., Bell, J., and Robinson, M. (1993). KIM185, a monoclonal antibody to CD18 which induces a change in the conformation of CD18 and promotes both LFA-1- and CR3-dependent adhesion. Eur. J. Immunol. 23, 2217–2222.

Arnaout, M.A. (1990). Leukocyte adhesion molecules deficiency: its structural basis, pathophysiology and implications for modulating the inflammatory response. Immunol. Rev. *114*, 145–180.

Arroyo, A.G., Campanero, M.R., Sanchez-Mateos, P., Zapata, J.M., Ursa, M.A., Delpozo, M.A., and Sanchez Madrid, F. (1994). Induction of tyrosine phosphorylation during ICAM-3 and LFA-1-mediated intercellular adhesion, and its regulation by the CD45 tyrosine phosphatase. J. Cell Biol. *126*, 1277–1286.

Beatty, P.G., Ledbetter, J.A., Martin, P.J., Price, T.H., and Hansen, J.A. (1983). Definition of a common leukocyte cell-surface antigen (Lp95–150) associated with diverse cell-mediated immune functions. J. Immunol. *131*, 2913–2918.

Berlin, C., Bargatze, R.F., Campbell, J.J., Vonandrian, U.H., Szabo, M.C., Hasslen, S.R., Nelson, R.D., Berg, E.L., Erlandsen, S.L., and Butcher, E.C. (1995). alpha 4 Integrins mediate lymphocyte attachment and rolling under physiologic flow. Cell *80*, 413–422.

Burn, P., Kupfer, A., and Singer, S.J. (1988). Dynamic membrane-cytoskeletal interactions: specific association of integrin and talin arises in vivo after phorbol ester treatment of peripheral blood lymphocytes. Proc. Natl. Acad. Sci. USA. *85*, 497–501.

Crowe, D.T., Chiu, H., Fong, S., and Weissman, I.L. (1994). Regulation of the avidity of integrin alpha(4)beta(7) by the beta(7) cytoplasmic domain. J. Biol. Chem. *269*, 14411–14418.

de Fougerolles, A.R., Klickstein, L.B., and Springer, T.A. (1993). Cloning and expression of intercellular adhesion molecule 3 reveals strong homology to other immunoglobulin family counter-receptors for lymphocyte function-associated antigen 1. J. Exp. Med. *177*, 1187–1192.

de Fougerolles, A.R., and Springer, T.A. (1992). Intercellular adhesion molecule 3, a third adhesion counter-receptor for lymphocyte function-associated molecule 1 on resting lymphocytes. J. Exp. Med. *175*, 185–190.

Dransfield, I., Cabanas, C., Barrett, J., and Hogg, N. (1992a). Interaction of leukocyte integrins with ligand is necessary but not sufficient for function. J. Cell Biol. *116*, 1527–1535.

Dransfield, I., Cabanas, C., Craig, A., and Hogg, N. (1992b). Divalent cation regulation of the function of the leukocyte integrin LFA-1. J. Cell Biol. *116*, 219–226.

Dransfield, I., and Hogg, N. (1989). Regulated expression of Mg^{2+} binding epitope on leukocyte integrin alpha subunits. EMBO J. *8*, 3759–3765.

Dustin, M.L. (1990). Two-way signalling through the LFA-1 lymphocyte adhesion receptor. Bioessays *12*, 421–427.

Dustin, M.L., and Springer, T.A. (1989). T-cell receptor cross-linking transiently stimulates adhesiveness through LFA-1. Nature *341*, 619–624.

Erle, D.J., Ruegg, C., Sheppard, D., and Pytela, R. (1991). Complete amino acid sequence of an integrin beta subunit (beta 7) identified in leukocytes. J. Biol. Chem. *266*, 11009–11016.

Fawcett, J., Holness, C.L., Needham, L.A., Turley, H., Gatter, K.C., Mason, D.Y., and Simmons, D.L. (1992). Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. Nature *360*, 481–484.

Figdor, C.G., van Kooyk, Y., and Keizer, G.D. (1990). On the mode of action of LFA-1. Immunol. Today *11*, 277–280.

Hayashi, Y., Haimovich, B., Reszka, A., Boettiger, D., and Horwitz, A. (1990). Expression and function of chicken integrin beta 1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. J. Cell Biol. *110*, 175–184.

Hemler, M.E. (1988). Adhesive protein receptors on hematopoietic cells. Immunol. Today *9*, 109–113.

Hemler, M.E. (1990). VLA proteins in the integrin family: structures, functions, and their role on leukocytes. Annu. Rev. Immunol. *8*, 365–400.

Hemler, M.E., Crouse, C., Takada, Y., and Sonnenberg, A. (1988). Multiple very late antigen (VLA) heterodimers on platelets. Evidence for distinct VLA-2, VLA-5 (fibronectin receptor), and VLA-6 structures. J. Biol. Chem. *263*, 7660–7665.

Hemler, M.E., Elices, M.J., Parker, C., and Takada, Y. (1990). Structure of the integrin VLA-4 and its cell-cell and cell-matrix adhesion functions. Immunol. Rev. *114*, 45–65.

Hemler, M.E., Huang, C., and Schwarz, L. (1987). The VLA protein family. Characterization of five distinct cell surface heterodimers each with a common 130,000 molecular weight beta subunit. J. Biol. Chem. *262*, 3300–3309.

Hemler, M.E., Sanchez Madrid, F., Flotte, T.J., Krensky, A.M., Burakoff, S.J., Bhan, A.K., Springer, T.A., and Strominger, J.L. (1984). Glycoproteins of 210,000 and 130,000 m.w. on activated T cells: cell distribution and antigenic relation to components on resting cells and T cell lines. J. Immunol. *132*, 3011–3018.

Hibbs, M.L., Jakes, S., Stacker, S.A., Wallace, R.W., and Springer, T.A. (1991a). The cytoplasmic domain of the integrin lymphocyte function-associated antigen 1 beta subunit: sites required for binding to intercellular adhesion molecule 1 and the phorbol ester-stimulated phosphorylation site. J. Exp. Med. *174*, 1227–1238.

Hibbs, M.L., Xu, H., Stacker, S.A., and Springer, T.A. (1991b). Regulation of adhesion of ICAM-1 by the cytoplasmic domain of LFA-1 integrin beta subunit. Science *251*, 1611–1613.

Hildreth, J.E. and August, J.T. (1985). The human lymphocyte function-associated (HLFA) antigen and a related macrophage differentiation antigen (HMac-1): functional effects of subunit-specific monoclonal antibodies. J. Immunol. *134*, 3272–3280.

Hughes, P.E., O'Toole, T.E., Ylanne, J., Shattil, S.J., and Ginsberg, M.H. (1995). The conserved membrane-proximal region of an integrin cytoplasmic domain specifies ligand binding affinity. J. Biol. Chem. *270*, 12411–12417.

Hynes, R.O. (1987). Integrins: a family of cell surface receptors. Cell *48*, 549–554.

Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*, 11–25.

Kanner, S.B., Grosmaire, L.S., Ledbetter, J.A., and Damle, N.K. (1993). Beta 2-integrin LFA-1 signaling through phospholipase C-gamma 1 activation. Proc. Natl. Acad. Sci. USA *90*, 7099–7103.

Keizer, G.D., Borst, J., Figdor, C.G., Spits, H., Miedema, F., Terhorst, C., and De Vries, E. (1985). Biochemical and functional characteristics of the human leukocyte membrane antigen family LFA-1, Mo-1 and p150,95. Eur. J. Immunol. *15*, 1142–1147.

Keizer, G.D., te Velde, A.A., Schwarting, R., Figdor, C.G., and de Vries, J.E. (1987). Role of p150,95 in adhesion, migration, chemotaxis and phagocytosis of human monocytes. Eur. J. Immunol. *17*, 1317–1322.

Keizer, G.D., Visser, W., Vliem, M., and Figdor, C.G. (1988). A monoclonal antibody (NKI-L16) directed against a unique epitope on the alpha-chain of human leukocyte function-associated antigen 1 induces homotypic cell-cell interactions. J. Immunol. *140*, 1393–1400.

Kurzinger, K., Reynolds, T., Germain, R.N., Davignon, D., Martz, E., and Springer, T.A. (1981). A novel lymphocyte function-associated antigen (LFA-1): cellular distribution, quantitative expression, and structure. J. Immunol. *127*, 596–602.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Landis, R.C., Bennett, R.I., and Hogg, N. (1993). A novel LFA-1 activation epitope maps to the I domain. J. Cell Biol. *120*, 1519–1527.

Lollo, B.A., Chan, K.W., Hanson, E.M., Moy, V.T., and Brian, A.A. (1993). Direct evidence for two affinity states for lymphocyte function-associated antigen 1 on activated T cells (published erratum appears in J. Biol. Chem. *269*, 10184, 1994). J. Biol. Chem. *268*, 21693–21700.

Lub, M., van Kooyk, Y., and Figdor, C.G. (1995). Ins and outs of LFA-1. Immunol. Today *16*, 479–483.

Marcantonio, E.E., Guan, J.L., Trevithick, J.E., and Hynes, R.O. (1990). Mapping of the functional determinants of the integrin beta 1 cytoplasmic domain by site-directed mutagenesis. Cell Regul. *1*, 597–604.

Marlin, S.D., and Springer, T.A. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell *51*, 813–819.

Martz, E. (1987). LFA-1 and other accessory molecules functioning in adhesions of T and B lymphocytes. Hum. Immunol. *18*, 3–37.

Mauro, L.J., and Dixon, J.E. (1994). "Zip codes" direct intracellular protein tyrosine phosphatases to the correct cellular "address." Trends Biochem. Sci. *19*, 151–155.

Miyamoto, S., Akiyama, S.K., and Yamada, K.M. (1995). Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science *267*, 883–885.

O'Toole, T.E., Ylanne, J., and Culley, B.M. (1995). Regulation of integrin affinity states through an NPXY motif in the beta subunit cytoplasmic domain. J. Biol. Chem. *270*, 8553–8558.

Ortlepp, S., Stephens, P.E., Hogg, N., Figdor, C.G., and Robinson, M.K. (1995). Antibodies that activate beta 2 integrins can generate different ligand binding states. Eur. J. Immunol. *25*, 637–643.

Pavalko, F.M., and LaRoche, S.M. (1993). Activation of human neutrophils induces an interaction between the integrin β_2-subunit (CD18) and the actin binding protein α-actinin. J. Immunol. *151*, 3795–3807.

Peter, K., and O'Toole, T.E. (1995). Modulation of cell adhesion by changes in alpha(L)beta(2) (LFA-1, CD11a/CD18) cytoplasmic domain/cytoskeleton interaction. J. Exp. Med. *181*, 315–326.

Reszka, A.A., Hayashi, Y., and Horwitz, A.F. (1992). Identification of amino acid sequences in the integrin beta 1 cytoplasmic domain implicated in cytoskeletal association. J. Cell Biol. *117*, 1321–1330.

Robinson, M.K., Andrew, D., Rosen, H., Brown, D., Ortlepp, S., Stephens, P., and Butcher, E.C. (1992). Antibody against the Leu-CAM beta-chain (CD18) promotes both LFA-1- and CR3-dependent adhesion events. J. Immunol. *148*, 1080–1085.

Rothlein, R., and Springer, T.A. (1986). The requirement for lymphocyte function-associated antigen 1 in homotypic leukocyte adhesion simulated by phorbol ester. J. Exp. Med. *163*, 1132–1149.

Ruegg, C., Postigo, A.A., Sikorski, E.E., Butcher, E.C., Pytela, R., and Erle, D.J. (1992). Role of integrin alpha 4 beta 7/alpha 4 beta P in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. J. Cell Biol. *117*, 179–189.

Sanchez Madrid, F., Krensky, A.M., Ware, C.F., Robbins, E., Strominger, J.L., Burakoff, S.J., and Springer, T.A. (1982). Three distinct antigens associated with human T-lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3. Proc. Natl. Acad. Sci. USA *79*, 7489–7493.

Solowska, J., Guan, J.L., Marcantonio, E.E., Trevithick, J.E., Buck, C.A., and Hynes, R.O. (1989). Expression of normal and mutant avian integrin subunits in rodent cells (published erratum appears in J. Cell Biol. *109*, 1187, 1989). J. Cell Biol. *109*, 853–861.

Springer, T.A. (1990). Adhesion receptors of the immune system. Nature *346*, 425–434.

Staunton, D.E., Dustin, M.L., and Springer, T.A. (1989). Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature *339*, 61–64.

Takada, Y., Huang, C., and Hemler, M.E. (1987). Fibronectin receptor structures in the VLA family of heterodimers. Nature *326*, 607–609.

van de Wiel-van Kemenade, E., van Kooyk, Y., De Boer, A.J., Huijbens, R.J.F., Weder, P., Van de Kasteele, W., Melief, C.J.M., and Figdor, C.G. (1992). Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA. J. Cell Biol. *117*, 461–470.

van Kooyk, Y., van de Wiel-van Kemenade, P., Weder, P., Kuijpers, T.W., and Figdor, C.G. (1989). Enhancement of LFA-1-mediated cell adhesion by triggering through CD2 or CD3 on T lymphocytes. Nature *342*, 811–813.

van Kooyk, Y., Weder, P., Heije, K., and Figdor, C.G. (1994). Extracellular Ca^{2+} modulates leukocyte function-associated antigen-1 cell surface distribution on T lymphocytes and consequently affects cell adhesion. J. Cell Biol. *124*, 1061–1070.

Vazeux, R., Hoffman, P.A., Tomita, J.K., Dickinson, E.S., Jasman, R.L., Stjohn, T., and Gallatin, W.M. (1992). Cloning and characterization of a new intercellular adhesion molecule ICAM-R. Nature *360*, 485–488.